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Cyclopropane fatty acid synthase mutants of probiotic human-derived *Lactobacillus reuteri* are defective in TNF inhibition

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Abbreviations: CFA, cyclopropane fatty acid; TNF, tumor necrosis factor; LPS, lipopolysaccharide; FAME, fatty acid methyl ester; ANOVA, analysis of variance; LMW, low molecular weight

Although commensal microbes have been shown to modulate host immune responses, many of the bacterial factors that mediate immune regulation remain unidentified. Select strains of human-derived *Lactobacillus reuteri* synthesize immunomodulins that potently inhibit production of the inflammatory cytokine TNF. In this study, genetic and genomic approaches were used to identify and investigate *L. reuteri* genes required for human TNF immunomodulatory activity. Analysis of membrane fatty acids from multiple *L. reuteri* strains cultured in MRS medium showed that only TNF inhibitory strains produced the cyclopropane fatty acid (CFA) lactobacillic acid. The enzyme cyclopropane fatty acid synthase is required for synthesis of CFAs such as lactobacillic acid, therefore the *cfa* gene was inactivated and supernatants from the *cfa* mutant strain were assayed for TNF inhibitory activity. We found that supernatants from the wild-type strain, but not the *cfa* mutant, suppressed TNF production by activated THP-1 human monocytoid cells. Although this suggested a direct role for lactobacillic acid in immunomodulation, purified lactobacillic acid did not suppress TNF at physiologically relevant concentrations. We further analyzed TNF inhibitory and TNF non-inhibitory strains under different growth conditions and found that lactobacillic acid production did not correlate with TNF inhibition. These results indicate that *cfa* indirectly contributed to *L. reuteri* immunomodulatory activity and suggest that other mechanisms, such as decreased membrane fluidity or altered expression of immunomodulins, result in the loss of TNF inhibitory activity. By increasing our understanding of immunomodulation by probiotic species, beneficial microbes can be rationally selected to alleviate intestinal inflammation.

Introduction

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts.[1,2] Many probiotic strains are now marketed to consumers and include organisms such as lactobacilli, streptococci, bifidobacteria, *Escherichia coli* Nissle 1917, and the yeast *Saccharomyces cerevisiae* strain, *"Saccharomyces boulardii"*.[3] In general, probiotics are considered safe for human consumption based on previous clinical trials, epidemiological studies and historical usage in fermented foods.[4,5] A subset of these probiotics have been derived from commensal bacteria indigenous to *Homo sapiens*. One such indigenous Lactobacillus species, *Lactobacillus reuteri*, includes a variety of strains derived from human breast milk and the gastrointestinal tract. The mechanisms by which commensal-derived probiotics promote health and combat diseases are poorly understood. Possible mechanisms include improving nutrient absorption, pathogen exclusion, strengthening intestinal barrier function and regulation of the immune system.[6] A better understanding of how probiotics influence health is critical to fully optimize the integrative physiology of commensal microbial communities and mammalian hosts.

Recent probiotic research demonstrates the ability of these bacteria to alter the functions of the immune system.[7] Several probiotics, termed immunoprobiotics, produce key immunomodulins that modulate the host inflammatory response. The bacterial products responsible for the effects on inflammation are largely undefined although recent work has suggested the cell

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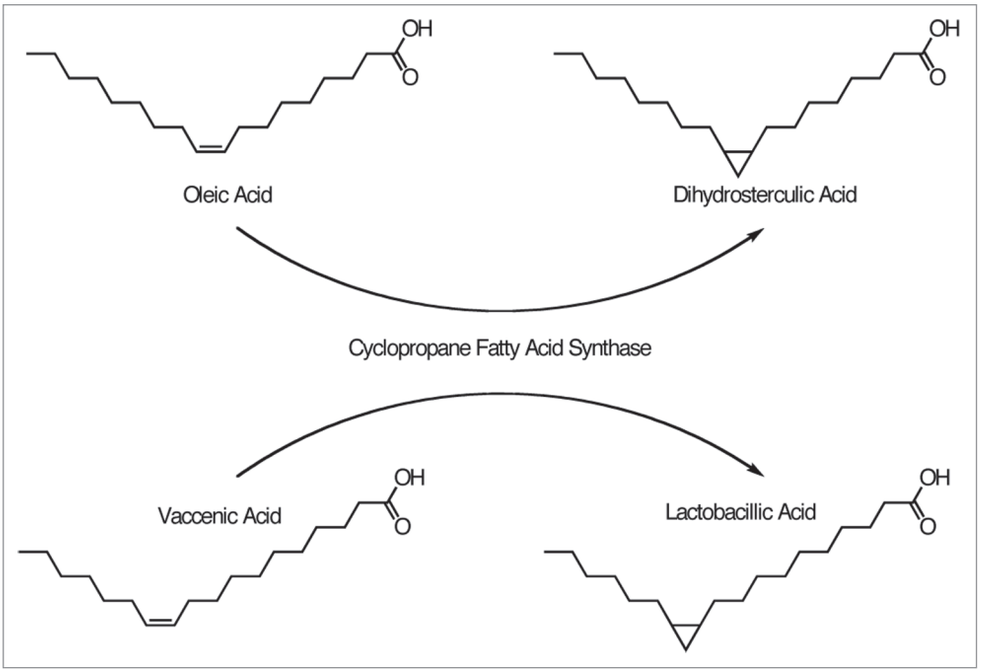

Figure 1. Synthesis of cyclopropane fatty acids in bacteria. Cyclopropane fatty acid synthase converts oleic acid and vaccenic acid into dihydrosterculic acid and lactobacillic acid, respectively.[14]

wall, DNA and exopolysaccharides as possible immunomodulatory factors.[7-10] Immunoprobiotics are potential therapies for various immune-mediated disorders such as Crohn disease.[11] The inflammatory cytokine TNF, produced by monocytes and macrophages, is a key mediator of intestinal inflammation. Indeed, select *L. reuteri* strains inhibit TNF production by human monocytoid THP-1 cells and monocytes isolated from patients with Crohn disease.[10,12] Strains of *L. reuteri* and *L. paracasei* capable of inhibiting TNF and IL-12 production in tissue explants were able to reduce inflammation in a *Helicobacter hepaticus*-induced murine model of inflammatory bowel disease.[10] How probiotics mediate these immunomodulatory effects is a vigorous area of research today.

Cyclopropane fatty acids (CFAs) are fatty acids that contain a cyclopropane ring. The first CFA was identified as cis-11,12-methylene octadecanoic acid or lactobacillic acid, in *L. arabinosus*.[13] Additional CFAs, such as dihydrosterculic acid, have been identified in gram-negative and gram-positive bacteria.[14] As shown in **Figure 1**, lactobacillic and dihydrosterculic acids are produced from the precursors, vaccenic acid and oleic acid respectively, by the enzyme cyclopropane fatty acid synthase. Lactobacillic and dihydrosterculic acids are 19 carbon CFAs that differ only in the position of the cyclopropane ring. During synthesis, CFA synthase adds a methylene group across the double bond forming a three-carbon ring using S-adenosylmethionine as a methyl donor.[14] While our knowledge of how CFAs are produced has greatly increased in the past decade, the physiological role(s) of CFAs are poorly understood. CFAs may stabilize the cellular membrane, allowing bacteria to survive exposure to adverse conditions such as extreme acid shocks and repeated freeze/thaw cycles.[15,16] Recent work indicates that the ability to survive acid stress is due to the fact that CFAs reduce membrane permeability to H^+ and increase the ability of cells to extrude protons.[17]

Some probiotic *L. reuteri* strains have the ability to produce small (<3 kDa), secreted factors that suppress lipopolysaccharide (LPS)-induced TNF production in primary monocytes, myeloid cell lines and macrophages.[12,18] This activity is strain-specific. We have identified three strains of *L. reuteri* (ATCC PTA 6475, ATCC PTA 4659 and ATCC PTA 5289) that secrete one or more compounds that exhibit potent immunomodulatory activity against human TNF production in activated macrophages. Three additional strains (*L. reuteri* ATCC 55730, DSM 17938 and CF48-3A) were incapable of reducing TNF production.[18] The inhibitory effect on TNF production is found in culture supernatants of cells grown into stationary phase and is not detected from culture supernatants of exponentially growing *L. reuteri*. The goal of this study was to identify TNF inhibitory compounds produced upon entry into stationary phase or genes involved in regulating these compounds using a combined genomics and genetic approach. Here we show that a *L. reuteri* mutant defective in producing cyclopropane fatty acids is unable to suppress TNF production from activated human myeloid cells.

Results

The cyclopropane fatty acid, lactobacillic acid, is specifically produced in human-derived *L. reuteri* strains capable of inhibiting human TNF. The membrane fatty acid profiles of several human-derived *L. reuteri* strains were compared in order to identify potential fatty acids that are produced specifically by TNF inhibitory strains. We analyzed and compared the membrane fatty acid profiles of three *L. reuteri* strains capable of inhibiting TNF production (ATCC PTA 6475, ATCC PTA 4659 and ATCC PTA 5289) and two *L. reuteri* strains incapable of downregulating TNF production (ATCC 55730 and CF48-3A). These five strains were grown under conditions in which potent immunomodulatory activity is present (stationary phase in MRS medium under anaerobic conditions[12]). Fatty acid methyl ester analysis (FAMES) was performed to determine the membrane fatty acid content. We found the fatty acid profiles of the TNF inhibitory strains were more similar to each other and distinct from that of TNF non-inhibitory strains (**Fig. 2**). The relative level of most fatty acids differed by less than 2-fold between the TNF non-inhibitory and TNF inhibitory strains. However, the

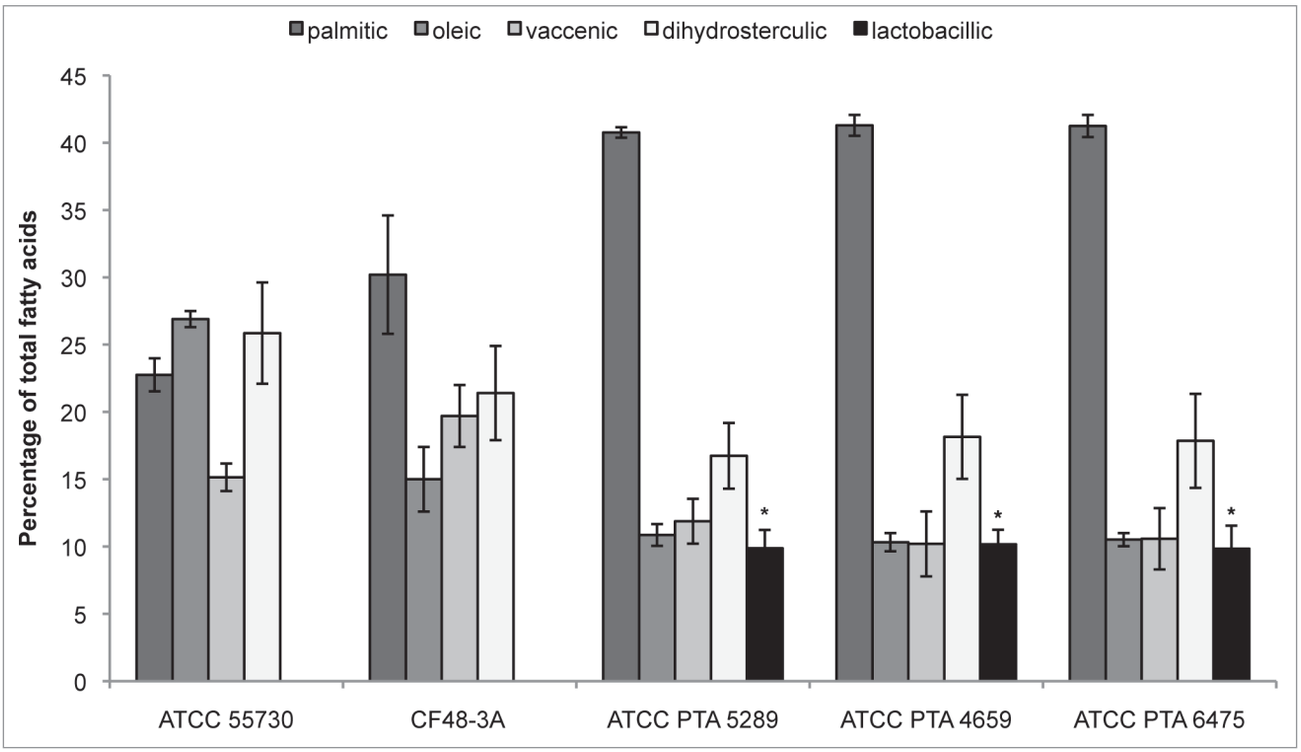

Figure 2. FAME analysis of TNF inhibitory and TNF non-inhibitory strains. Five *L. reuteri* strains that differ in their ability to downregulate human TNF were grown to stationary phase in MRS and FAME analysis was performed. ATCC 55730 and CF48-3A are TNF non-inhibitory strains while ATCC PTA 6475, ATCC PTA 4659 and ATCC PTA 5289 are TNF inhibitory strains. The level of each fatty acid produced in the strains is indicated as a percentage of the total membrane fatty acids on the Y-axis. The identity of each fatty acid is indicated by color in the figure. * denotes lactobacillic acid.

TNF inhibitory strains contained a cyclopropane fatty acid, lactobacillic acid, which was not detectable in TNF non-inhibitory strains. This finding identified lactobacillic acid as a potential candidate immunomodulin produced by *L. reuteri* strains ATCC PTA 6475, ATCC PTA 4659 and ATCC PTA 5289.

The production of lactobacillic acid correlates with the appearance of TNF inhibitory activity. To further investigate a possible role of lactobacillic acid in immunomodulation, we asked if the production of lactobacillic acid increased upon entry into stationary phase. If lactobacillic acid is part of the TNF inhibitory activity, then its production should correlate with the appearance of TNF inhibitory activity. We therefore monitored the production of lactobacillic acid in *L. reuteri* ATCC PTA 6475 during different phases of growth (**Fig. 3**). Lactobacillic acid was only detected during stationary phase, directly correlating with the appearance of TNF inhibitory activity.

Disruption of the gene encoding cyclopropane fatty acid synthase (*cfa)* renders *L. reuteri* incapable of producing cyclopropane fatty acids. We disrupted the only gene in the *L. reuteri* ATCC PTA 6475 genome with significant similarity to cyclopropane fatty acid synthase and monitored the production of lactobacillic acid. Previous work in *E. coli* had demonstrated that deletion of *cfa* resulted in the complete loss of cyclopropane fatty acids.[15,16] We cloned an internal fragment of the *cfa* gene into plasmid pORI28 and generated an insertion mutation of *cfa* in *L. reuteri* ATCC PTA 6475 (see Materials and Methods for details). The resulting strain was used to identify if mutation of the *cfa* gene would eliminate cyclopropane fatty acid production. We found that the resulting strain, PRB173 (*cfa::pORI28,* referred to as Δ*cfa* hereafter) was no longer able to produce the two cyclopropane fatty acids detected in *L. reuteri*, lactobacillic acid and dihyrdosterculic acid, as expected (see **Fig. 5**).

Extracellular low molecular weight (LMW) factors from a mutant defective in cyclopropane fatty acid production no longer inhibit human TNF production by activated monocytes. To address the possible role of lactobacillic acid on TNF production, we compared the effects of conditioned media made from wild-type cells (*L. reuteri* ATCC PTA 6475) and the *cfa*::pORI28 mutant (PRB173) on the ability of activated human monocytes to produce TNF. We analyzed immunomodulation using two different growth conditions as previously described in reference 12 and 18. *L. reuteri* strains were grown as planktonic cultures in the rich medium MRS (**Fig. 4A**) or the defined medium LDMIII (**Fig. 4B**), and conditioned media were prepared. Human derived THP-1 monocytes were activated by the addition of the TLR2 agonist, PCK and the production of human TNF was monitored by quantitative ELISA. In MRS medium, ATCC PTA 6475 showed a ~90% reduction in the release of TNF from monocytes, while the PRB173 (*cfa*::pORI28) completely lost the ability to suppress TNF production (**Fig. 4A**). Similar results were obtained when using LDMIII medium (**Fig. 4B**), which is a semi-defined medium being used to identify LMW immunomodulatory factors produced by *L. reuteri. L. reuteri* ATCC PTA 6475 reduced TNF production by ~95%,

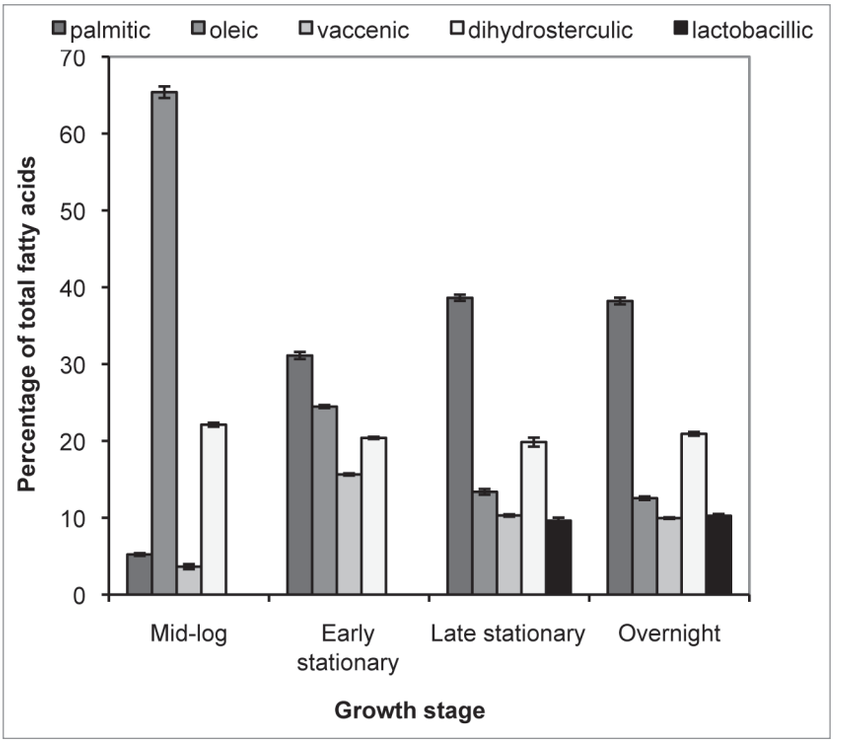

Figure 3. The appearance of lactobacillic acid in late stationary phase cultures of *L. reuteri* ATCC PTA 6475 correlates with the appearance of TNF inhibitory activity by cell-free supernatants. Cultures of *L. reuteri* ATCC PTA 6475 were grown in MRS broth overnight at 37°C. Samples were collected for FAME analysis at different stages of growth. Experiments were performed in triplicate; error bars represent standard deviations. The relative quantities of each fatty acid produced in the strains are indicated as a percentage of the total membrane fatty acids on the Y-axis. The identity of each fatty acid is indicated by color in the figure.

while the *cfa* mutant again completely eliminated the ability of *L. reuteri* to suppress TNF production. *L. reuteri* strain DSM 17938 grown in LDMIII medium lacked the ability to suppress TNF (**Fig. 4B**), again highlighting differences among human-derived commensal bacteria in their relative abilities to suppress TNF production. We observed no suppression of TNF by conditioned medium produced by *L. reuteri* ATCC 55730 (parent strain of DSM 17938,[19]), as has been previously described (data not shown).[12,18] To insure that the mutagenesis system used to disrupt the *cfa* gene did not yield global effects that included differences in immunomodulatory capacity, we tested two different mutants containing disruptions in genes unrelated to fatty acid metabolism. Neither mutation had any effect on the ability of *L. reuteri* ATCC PTA 6475 to inhibit TNF production, indicating that the mutagenesis system does not affect TNF levels (data not shown). These results demonstrate that *L. reuteri* mutants defective in cyclopropane fatty acid synthase activity are unable to suppress TNF production.

Lactobacillic acid is produced in both TNF inhibitory and TNF non-inhibitory strains when grown in LDMIII medium. The results above suggested that lactobacillic acid might be one factor responsible for the suppression of TNF production. We wanted to confirm that production of lactobacillic acid occurs only in TNF inhibitory strains grown in LDMIII medium as was originally observed in MRS medium. Surprisingly, FAMES analysis of *L. reuteri* ATCC PTA 6475 and *L. reuteri* ATCC 55730 showed that both strains contained similar amounts of lactobacillic acid when grown in LDMIII medium (**Fig. 5**), in contrast to the results of these strains grown in MRS medium (**Fig. 2**). As expected, the PRB173 Δ*cfa* strain did not produce detectable levels of lactobacillic acid in either medium. The presence of lactobacillic acid in all *L. reuteri* strains, despite the strain differences in TNF inhibition, demonstrates that lactobacillic acid does not have a direct role in suppressing TNF production. Another significant difference observed in the membrane fatty acid analysis between strains grown in LDMIII was the vaccenic acid content. Vaccenic acid was reduced approximately 5-fold in the ATCC PTA 6475 strain when compared to ATCC 55730 or the Δ*cfa* mutant (ATCC PTA 6475 background) (**Fig. 5**). This suggests that vaccenic acid may stimulate TNF production or somehow counteract TNF inhibition. However, experiments using purified vaccenic acid and lactobacillic acid showed they had no effect on TNF production at physiologically relevant concentrations ranging from 0.05 uM to 5 uM (data not shown). These data suggest an indirect role for *cfa,* cyclopropane fatty acids, and/or their precursors in immunomodulation.

Comparative transcriptome profiling of *L. reuteri* ATCC PTA 6475 and the congenic Δ*cfa* mutant strain PRB173. The data above support an indirect effect model in which the Δ*cfa* mutant is affecting the production or extracellular release of a LMW inhibitor of TNF production. To address genome-wide physiological changes in the Δ*cfa* strain, DNA microarrays were used to assess transcriptional changes between *L. reuteri* ATCC PTA 6475 and the PRB173 Δ*cfa* mutant strain. Both strains were grown in MRS for twenty-four hours to simulate growth conditions used for immunomodulation studies and microarrays were performed (see Materials and Methods). Fifty-six genes were differentially expressed by more than 1.5-fold when the two strains were compared. Thirty-six genes were decreased in expression (**Table 2**) and 20 genes were increased in expression (**Table 3**) in the *cfa*-deficient strain PRB173. Genes that are decreased in expression are possible targets for mediating the indirect effect of the Δ*cfa* mutation on human TNF production. A putative cyclase family protein gene (NT01LR1833) was the most downregulated gene (by 10.1-fold) in the *cfa* strain. Interestingly, this gene is present in anti-inflammatory strains of *L. reuteri* but absent from immunostimulatory strains. The largest class of affected genes (17 total) in the *cfa*-deficient strain was comprised of phage related genes and these genes were divided between two operons. In the Δ*cfa* strain, the majority of the genes increased in expression are predicted

to be involved in energy metabolism (7 genes) or hypothetical proteins (6 genes). Three genes increased in expression in the Δ*cfa* mutant are involved in the osmotic stress response and acid stress response, pathways known to be affected by CFAs in bacteria.

Discussion

The era of microbiome research has focused attention on functionally diverse microbial communities with important implications for the systems biology of mammals and immunology. The ability of commensal bacteria and probiotics to affect the immune system of the host is likely to be an important strategy for microbes to impact human physiology and promote health-associated microbe:host interactions. Several gastrointestinal disorders, including Crohn disease, include intestinal inflammation as a hallmark of human disease and therapies that target pro-inflammatory cytokines such as TNF have been effective in providing symptomatic relief.[20] Thus, commensally derived probiotics that can downregulate pro-inflammatory cytokines or interfere with cytokine receptor signaling offer a means of localized delivery of TNF inhibitory compounds. In this study, we have identified the activity of the bacterial enzyme cyclopropane fatty acid synthase as being indirectly involved in the anti-inflammatory effect of *L. reuteri* ATCC PTA 6475. Our data suggest that neither of two cyclopropane fatty acids, lactobacillic acid or dihydrosterculic acid, are directly responsible for mediating the repression of human TNF production. Our initial finding that the cyclopropane fatty acid lactobacillic acid is expressed only in immunomodulatory strains of *L. reuteri* when grown in MRS led us to investigate the role of cyclopropane fatty acid synthase, however, our results do not support a direct role for cyclopropane fatty acids in immunomodulation.

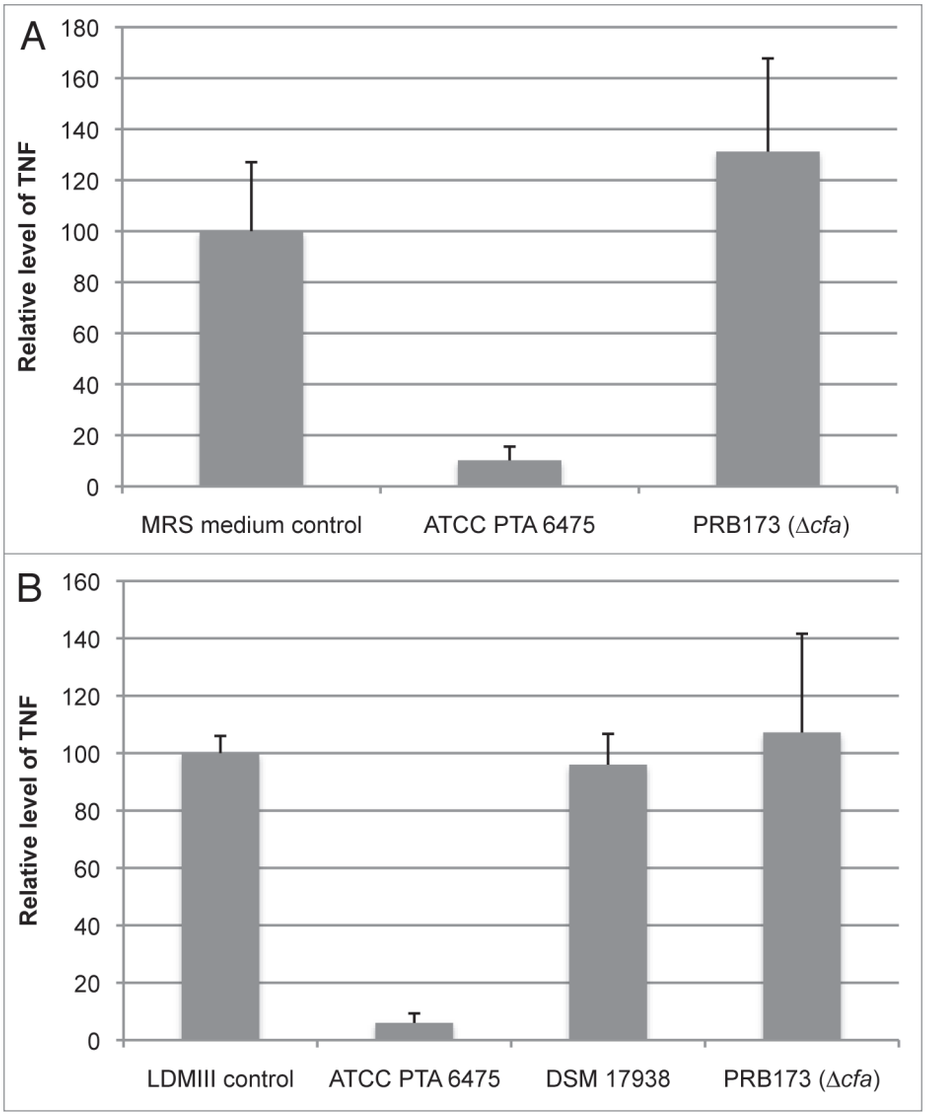

Figure 4. *L. reuteri* ATCC PTA 6475 deficient in cyclopropane fatty acid production is unable to suppress human TNF production by activated monocytes. Cell-free supernatants from *L. reuteri* strains grown in (A) MRS medium or (B) LDMIII medium were tested for the ability to inhibit activated THP-1 human monocytoid cells from producing TNF. TNF production (pg/ml) was monitored by ELISA and the data were normalized to the medium control, which was set at 100%. Strains tested were *L. reuteri* ATCC PTA 6475, PRB173 (Δ*cfa*) and *L. reuteri* DSM 17938. At least six independent experiments were performed and error bars indicate the standard deviation of the measurements. Using one-way ANOVA analysis, the relative quantities of TNF produced by wild-type *L. reuteri* ATCC PTA 6475 (in both MRS medium and LDMIII medium) were significantly different from that of medium control, PRB173 and *L. reuteri* DSM 17938 ($p < 0.001$). PRB173 and *L. reuteri* DSM 17938 were not significantly different than the medium control.

How might *L. reuteri* cells that are defective in CFA production be less able to suppress TNF activation? Our working hypothesis is that CFAs alter membrane composition and permeability. We speculate that the lack of CFAs changes the permeability of bacterial membranes, thereby reducing the ability of immunomodulin(s) to be secreted by anti-inflammatory strains of *L. reuteri*. Cyclopropane fatty acid synthase and CFAs have been shown to be involved in a number of stress responses including acid, ethanol and osmotic stress.[15-17,21] In each case, alteration of membrane fluidity and/or function is suggested as possible mechanisms of stress resistance. Testing of this hypothesis awaits the discovery of the molecules responsible for TNF suppression, which we are actively pursuing. Alternatively, disruption of *cfa* may yield an altered physiological response or changes in signaling pathways of *L. reuteri* that may impact the levels of

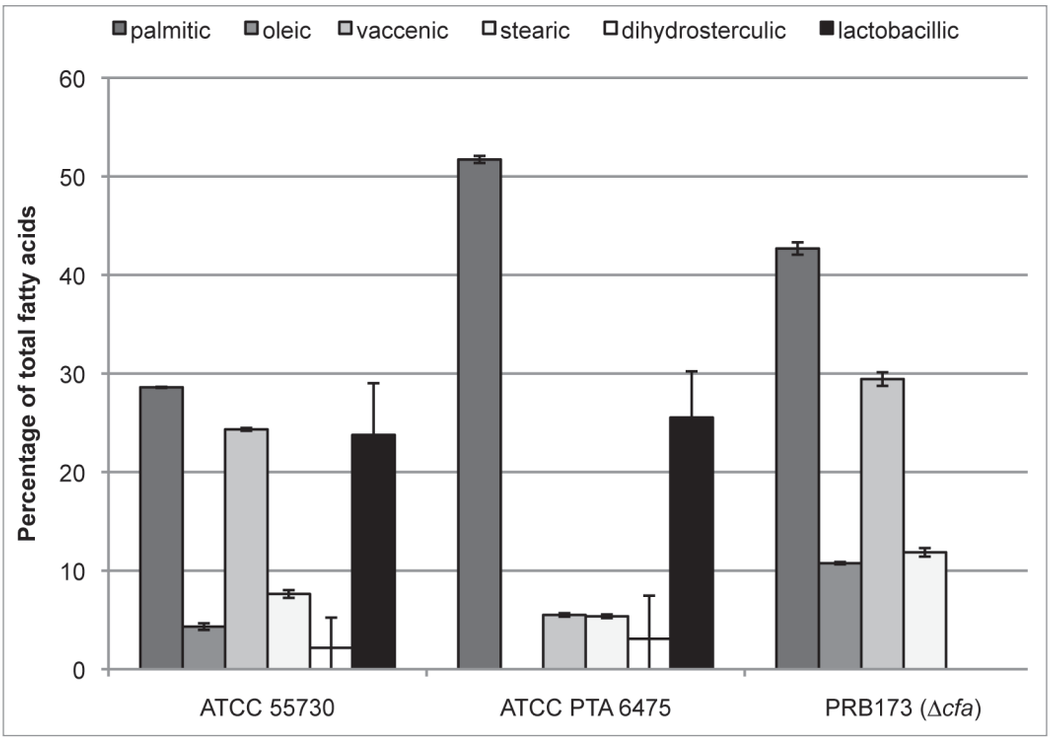

Figure 5. FAME analysis of TNF stimulatory and TNF inhibitory *L. reuteri* strains grown in LDMIII medium. *L. reuteri* cells that produced the cell free supernatants used in Figure 4B were subjected to FAME analysis. The percent of each fatty acid produced is indicated on the Y-axis. *L. reuteri* strains ATCC 55730, ATCC PTA 6475 and PRB173 (Δ*cfa*) are depicted on the X-axis.

immunomodulins produced. Our transcriptome analysis demonstrates that loss of CFAs impacts gene expression of *L. reuteri* ATCC PTA 6475. We are actively attempting to disrupt some of the genes altered in expression in the *cfa* mutant to test their effects on TNF production (see below).

We have identified 36 genes that are significantly reduced in expression when *cfa* is disrupted, and several genes have been identified as biologically attractive or intriguing possibilities. The most downregulated gene from this group is a putative metal dependant hydrolase/cyclase (NT01LR1833) that is found only in strains of *Lactobacillus reuteri* but not other lactobacilli. In fact this gene is only present in the genomes of TNF inhibitory strains and is lacking in other *L. reuteri* strains. We therefore disrupted NT01LR1833 and found no effect on TNF inhibitory activity, demonstrating that this putative metal dependent hydrolase/cyclase is not involved in immunomodulation (data not shown). This mutagenesis experiment highlights the limitations of candidate gene selection based entirely on the magnitude of changes in gene expression. We are currently targeting several other genes identified in the microarray experiment, including the ten phage like genes that are downregulated in the Δ*cfa* mutant, to identify which genes or pathways may contribute to anti-inflammatory effects by *L. reuteri*.

Our initial observation of fatty acid differences between anti-inflammatory strains of *L. reuteri* and strains lacking this activity highlighted the possibility that lactobacillic acid was a candidate immunomodulin. However, we have found that the lack of lactobacillic acid in *L. reuteri* ATCC 55730 was only observed in MRS medium, and the production of lactobacillic acid was similar between all strains when grown in LDMIII medium. This observation, as well as testing of purified lactobacillic acid for immunomodulation, ruled out a direct role for this CFA in this process. TNF inhibitory strains also showed differences in the levels of vaccenic acid (decreased) and palmitic acid (increased) when compared to non-inhibitory strains, however none of these changes directly correlated with the TNF inhibition data (**Fig. 4**).

Although our results do not support a direct role for CFAs in immunomodulation, it is worth noting that cyclopropane fatty acids can have effects on the immune response. The effects of lipid cyclopropanation on the immune response have recently been studied in *Mycobacterium tuberculosis*.[22] The disruption of cyclopropane ring formation in mycolic acids has a profound effect on how this pathogen interacts with the immune system. Deletion of the *cma2* gene, which encodes a cyclopropane synthase enzyme that adds trans-cyclopropane rings to mycolic acids, produces a hypervirulent strain in a mouse model of tuberculosis. Induction of pro-inflammatory cytokines in murine derived macrophages was more pronounced with the mutant strain, and this result could be mimicked with phenyl ether extracted lipids. These findings support the claim that trans-cyclopropanation of mycolic acids attenuates the host response to *M. tuberculosis*. Interestingly, deletion of the *pma* gene, which encodes another cyclopropane mycolic acid synthase in *M. tuberculosis* and is responsible for the synthesis of cis-cyclopropane rings in mycolic acids, had the opposite effect on virulence and *in vitro* inflammatory responses.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Description	Source
Bacterial strains		
L. reuteri ATCC 55730	Isolate from Peruvian mother's milk	Biogaia AB (Stockholm, Sweden)
L. reuteri DSM 17938	Derivative of ATCC 55730 lacking two plasmids	(Rosander, et al. 2008)
L. reuteri ATCC PTA 6475	Isolate from Finnish mother's milk	Biogaia AB (Stockholm, Sweden) (Egervarn, et al. 2007)
L. reuteri ATCC PTA 5289	Oral isolate from Japanese female	Biogaia AB (Stockholm, Sweden) (Egervarn, et al. 2007)
L. reuteri ATCC PTA 4659	Isolate from Finnish mother's milk	Biogaia AB (Stockholm, Sweden) (Egervarn, et al. 2007)
L. reuteri CF48-3A	Fecal isolate from Finnish child	Biogaia AB (Stockholm, Sweden) (Egervarn, et al. 2007)
L. reuteri PRB173	*L. reuteri* ATCC PTA 6475 with pORI28 inserted into the *cfa* gene (Δ*cfa*::pORI28)	This study
E. coli EC1000	contains chromosomal copy of the pWV01 *repA* gene; Kan^r	(Law, et al. 1995)
Plasmids		
pVE6007	Cm^r *repA*-positive temperature-sensitive derivative of pWV01	(Maguin, et al. 1992)
pORI28	Em^r *repA*-negative derivative of pWV01	(Law, et al. 1995)
pRB173	pORI28 + 258 bp insert from NT01LR1143	This study

This work indicates that cyclopropane fatty acids can influence pathogenicity and the host immune response.

We have demonstrated that select strains of *L. reuteri* lacking cyclopropane fatty acid synthase activity do not suppress production of the proinflammatory cytokine TNF by activated human monocytoid cells. The direct end products of cyclopropane fatty acid synthase activity in *L. reuteri*, lactobacillic and dihydrosterculic acid, are not directly responsible for the TNF inhibition, so we hypothesize that inactivation of *cfa* may alter cell permeability or expression of genes and associated signals required for immunomodulin synthesis or secretion. Through gene expression analysis, we have identified several potential immunomodulins and these candidates are being actively pursued. Understanding the genetic pathways required for production and secretion of potent immunomodulins will facilitate a deeper understanding of the functions of the human microbiome and allow for rapid identification of effective immunotherapeutic probiotics.

Materials and Methods

Key reagents, bacterial strains and mammalian cell lines. All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Polystyrene plates were obtained from Corning (Corning, NY). Polyvinylidene fluoride membrane filters of 0.22 μm pore size (Millipore, Bedford, MA) were used to yield *L. reuteri*-derived cell-free supernatants. Amicon Ultra-15 centrifugal filter unit with ultracel-3 membrane filters were used to size fractionate the supernatants. All bacterial strains and plasmids are described in **Table 1**. *L. reuteri* strains were cultured in deMan, Rogosa, Sharpe (MRS) (Difco, Franklin Lakes, NJ) or LDMIII media. Lactobacillic acid was purchased from Indofine Chemical Company, Inc., (Hillsbourough, NJ) or Cayman Chemicals (Ann Arbor, MI). Lactobacillic acid was resuspended in DMSO for TNF studies. For anaerobic culturing, an anaerobic chamber (1025 Anaerobic System, Forma Scientific, Waltham, MA) supplied with a mixture of 10% CO_2, 10% H_2 and 80% N_2 was used. THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. Erythromycin (10 μg/ml) was added to the culture for growth of PRB173.

Characterizing the fatty acid composition of *L. reuteri*. Ten mL of MRS was inoculated at a starting OD_{600} = 0.1 (~7 x 10^7 CFU). Bacteria were incubated for 24 h at 35°C in ambient atmosphere. Cells were pelleted (4,000x g, room temperature, 10 min) and the supernatants were discarded. MIDI labs (Newark, DE) performed fatty acid methyl ester (FAME) analysis of bacterial cells as previously described in reference 23. Briefly, bacterial pellets were resuspended in 15% NaOH (w/v), dissolved in 50% aqueous methanol (v/v) and heated for 30 min at 100°C. This reaction was cooled to ambient temperature and 3.25 N HCl in 45.8% methanol was added. The mixture was heated to 80°C for 10 min and cooled to ambient temperature. Hexane/methyl tertbutyl ether (1:1 v/v) was added. The organic phase was extracted and washed with 1.2% NaOH (w/v). The fatty acid extracts were analyzed on a Hewlett-Packard model HP5890 Series II gas chromatograph using the EUKARY method. Purified lactobacillic acid (phytomonic acid) was purchased from Cayman Chemicals and dihydrosterculic acid was purchased from Sigma. These were used as standards to ensure assignment of peaks to the correct CFA.

Mutagenesis strategy of *L. reuteri*. The cyclopropane fatty acid synthase (*cfa*) mutant was created using a previously described method in reference 24, with modifications for *L. reuteri*.[25,26] In short, a 258 basepair internal fragment from *cfa* (corresponding to basepairs 178–435) was cloned into pORI28

Table 2. *L. reuteri* genes downregulated in PRB173 (*cfa*$^-$)

Functional group and ORF	Description	Fold change
Hypothetical proteins		
NT01LR1833	putative cyclase family protein (Lreu_1082)	10.1
NT01LR1733	Conserved hypothetical protein (Lreu_1124)	4.2
NT01LR1334	conserved hypothetical protein (Lreu_1266) DNA repair ATPase	4.0
NT01LR2006	conserved hypothetical protein (Lreu_1097)	3.1
NT01LR2004	hypothetical protein (Lreu_1097)	2.7
NT01LR2005	conserved hypothetical protein (Lreu_1097)	2.7
Energy metabolism		
NT01LR2007	conserved hypothetical protein (N-acetylmuramoyl-L-alanine amidase, family 2) (Lreu_1094)	3.0
NT01LR1589	alcohol dehydrogenase, propanol-preferring (Lreu_1496)	2.5
glnA (NT01LR1234)	glutamine synthetase, type I (Lreu_1202)	2.0
Purines, pyrimidines, nucleosides and nucleotides		
pyrB (NT01LR1149)	aspartate carbamoyltransferase (Lreu_0123)	3.1
pyrF (NT01LR1146)	orotidine 5'-phosphate decarboxylase (Lreu_0126)	3.0
NT01LR1148	Dihydroorotase (Lreu_0124)	2.7
Protein synthesis		
tyrS (NT01LR1124)	tyrosyl-tRNA synthetase (Lreu_0148)	2.1
Transporters		
NT01LR1174	ABC-type polar amino acid transport system, ATPase component (glutamine transport ATP-binding protein GlnQ) (Lreu_0099)	4.0
NT01LR1660	permease for cytosine/purines, uracil, thiamine, allantoin (Lreu_0257)	2.1
Cellular processes		
NT01LR1192	primosomal protein DnaI (Lreu_1242)	6.7
NT01LR1096	modification methylase HemK [protein-(glutamine-N5) methyltransferase, release factor-specific] (Lreu_0453)	2.1
Phage and prophage related functions		
NT01LR0020	putative phage-associated protein (Lreu_1122)	4.1
NT01LR0005	hypothetical protein (Lreu_1107)	3.5
NT01LR0003	protein of unknown function (Lreu_1105)	3.5
NT01LR0002	Conserved hypothetical protein (Lreu_1104)	3.2
NT01LR0015	putative phage terminase, large subunit (Lreu_1117)	3.2
NT01LR0018	phage terminase, small subunit, putative, P27family (Lreu_1120)	3.1
NT01LR0001	Conserved hypothetical protein (Lreu_1104)	3.0
NT01LR0004	phage tape measure protein (Lreu_1106)	3.0
NT01LR0007	phage major tail protein (Lreu_1109)	2.9
NT01LR0006	conserved hypothetical protein (Lreu_1108)	2.9
NT01LR0011	phage qlrg family, putative dna packaging (Lreu_1113)	2.9
NT01LR0009	conserved hypothetical protein (Lreu_1111)	2.9
NT01LR0014	phage portal protein, HK97 family (Lreu_1116)	2.8
NT01LR0008	phage tail protein (Lreu_1110)	2.7
NT01LR0823	conserved domain protein (Lreu_1143)	2.5
NT01LR0010	phage head-tail adaptor, putative (Lreu_1112)	2.4
NT01LR0013	peptidase S14, ClpP (Lreu_1115)	2.3

Comparative transcriptome studies of *L. reuteri* PRB173 and ATCC PTA 6475 cultured 24 hr in MRS indicated that 48 genes were downregulated in the Cfa-deficient strain by at least 1.5-fold, $p < 0.05$.

Table 3. *L. reuteri* genes upregulated in PRB173 (*cfa*$^-$)

Functional group and ORF	Description	Fold change
Hypothetical proteins		
NT01LR0736	conserved hypothetical protein (Lreu_1604)	8.5
NT01LR0592	hypothetical protein CPA2 family monovalent cation:proton (H^+) antiporter-2 (Lreu_0181)	3.2
NT01LR0597	conserved hypothetical protein (Lreu_0177)	2.2
NT01LR0704	conserved protein with OsmC domain (osmotic induced protein) (Lreu_0041)	2.2
NT01LR0056	solo B3/4 domain (Lreu_1756)	2.1
NT01LR0343	methyltransferase (NLP/P60 protein) (Lreu_0925)	2.0
Transporters		
NT01LR1837	lactose permease (Lactose-proton symport) (Lreu_1086)	3.7
NT01LR0589	lr0281 (substrate-binding region of ABC-type glycine betaine transport system) (Lreu_0183)	2.7
NT01LR1409	collagen binding protein (extracellular solute-binding protein, family 3) (Lreu_296)	2.1
Cell Envelope		
NT01LR0675	integral membrane protein (Lreu_1582)	6.7
Energy metabolism		
NT01LR0703	D-lactate dehydrogenase (D-LDH) (D-specific D-2-hydroxyacid dehydrogenase) (Lreu_0042)	4.7
NT01LR0702	aromatic amino acid aminotransferase (putative aminotransferase A) (Lreu_0043)	3.5
NT01LR1836	beta-galactosidase 1 (Lreu_1085)	3.5
NT01LR0591	oxidoreductase (3-beta hydroxysteroid dehydrogenase/isomerase) (Lreu_0181)	3.4
NT01LR0841	malate dehydrogenase/L-lactate dehydrogenase (Lreu_0724)	2.4
carB	carbamoyl phosphate synthetase, subunit (Lreu_0085)	2.0
Protein synthesis		
trmU	tRNA(5-methylaminomethyl-2-thiouridylate)-methyltransferase (Lreu_0605)	3.0
smpB	SsrA-binding protein (Lreu_0395)	2.7
Cellular Processes		
xth-1	exodeoxyribonuclease III (Lreu_1311)	2.1
msrB	methionine-R-sulfoxide reductase (Lreu_0188)	2.0

Comparative transcriptome studies of PRB173 and ATCC PTA 6475 cultured for 24 hr in MRS indicated that 40 genes were upregulated in the Cfa-deficient strain by at least 1.5-fold, $p < 0.05$.

and used for insertional inactivation of the *cfa* gene. This construct (pRB173) was transformed into *E. coli* EC1000 and the transformed cells were grown in LB broth supplemented with 40 μg/mL kanamycin and 400 μg/mL erythromcyin. pRB173 was then extracted and transformed into *L. reuteri* ATCC PTA 6475 with pVE6007. *L. reuteri* cells containing both plasmids were grown in MRS broth containing 10 μg/mL chloramphenicol and 10 μg/mL erythromycin at the permissive temperature of 35°C for 18 h. The cultures were then shifted to the non-permissive temperature of 45°C and grown in the presence of erythromycin only. After several passages at 45°C to ensure loss of pVE6007, individual colonies were screened for integration of pRB173 at the desired location within the *cfa* gene by PCR.

Preparation of cell-free supernatants for immunomodulation studies. For planktonic cells, 10 mL of MRS or LDMIII medium was inoculated at a starting OD_{600} = 0.1 (~7 x 10^7 CFU) using 16–18 h *L. reuteri* cultures. Bacteria were incubated for 24 h at 37°C in anaerobic conditions. Cells were pelleted (4,000x g, room temperature, 10 min) and discarded. Supernatants were filter-sterilized through 0.22 μm PVDF filters and size fractionated through 3 kD filters. Aliquots of the 3 kD filtrate were vacuum-dried and re-suspended to the original volume using RPMI (1 mL). Serial dilutions were plated onto MRS medium to confirm that similar numbers of cells were used to produce conditioned medium under all growth conditions and mutant strain backgrounds.

Human TNF inhibition experiments. TNF assays were performed as previously described in reference 12. Briefly, cell-free supernatants from *L. reuteri* cultures (5% v/v) or serial dilutions of lactobacillic acid (2% v/v) were added to human THP-1 cells (approximately 5 x 10^4 cells) activated with two different Toll-like receptor agonists, either *E. coli* O127:B8 LPS (100 ng/mL) or Pam_3Cys-SKKKK x 3 HCl (EMC Microcollections) (100 ng/mL). After the addition of *L. reuteri* supernatants and LPS/Pam_3Cys-SKKKK, plates were incubated at 37°C with 5% CO_2 for 3.5 h. Trypan blue (Invitrogen, Carlsbad, CA) was

employed to ascertain cell viability. THP-1 cells were pelleted (1,500x g, 5 min, 4°C), and quantitative ELISAs (R&D Systems, Minneapolis, MN) were used to determine the amount of human TNF present in monocytoid cell supernatants.

Growth conditions for *L. reuteri* cultures used for microarray studies. Overnight cultures (14–16 h) of PRB173 (*cfa::pORI28*) and ATCC PTA 6475 cultured in MRS medium (supplemented with 10 μg/mL erythromycin for PRB173) were resuspended to a concentration of ~1.0 x 10^8 cells/mL in 10 mL of MRS broth. Cells were incubated for 24 h at 37°C in an anaerobic chamber. 10 mL of cold fixative solution (50% [v/v] cold methanol and MRS) was added to quickly stop transcription. The samples were then centrifuged (10 min at 1,500x g), and the pellets were stored at -80°C prior to RNA isolation. Experiments were performed in triplicate for each strain.

RNA isolation. Cells were resuspended in STE buffer (6.7% sucrose, 50 mM Tris [pH 8.0], 1 mM EDTA), harvested by centrifugation at 4,000x g for 10 min and resuspended in 100 μl of STE containing 5 μl of mutanolysin (5 U/μL). Cells were incubated at 37°C for 2 h. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), and DNA was removed by adding RNase-free DNase (Qiagen, Valencia, CA). RNA concentrations were measured at 260 nm with the ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The A_{260}/A_{280} ratio was measured to check the relative purity of the RNA. RNA samples were fractionated by 1% agarose gel electrophoresis to verify that RNA was not degraded.

Microarray experiments and data analysis. Oligonucleotides (60-mers) were designed and synthesized for 1,966 open reading frames from a draft genome sequence of *L. reuteri* ATCC PTA 6475. For expression analyses, three biological replicates were performed with dye-swap experiments. Following mRNA isolation, cDNA synthesis, labeling and hybridization were performed essentially as previously described in reference 26 and 27. Information regarding the microarray platform can be found at the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under GEO platform no. GPL7541. GenePix Pro 4.0.12 software was utilized for image analysis of the microarrays. Normalization within arrays and between arrays was performed by applying the Loess algorithm[28] using the Limma package[29] in R (http://www.r-project.org). Normalized intensities were used for further analysis. The average signal intensities of three biological replicates were calculated in order to compare the relative gene expression of mutant and wild type strains. The statistical significance of differences was calculated based on variation in biological duplicates, using the Bayes function in Limma (cross-probe variance estimation) and false discovery rate (FDR) adjustment of the p values. Only genes that were differentially expressed by least 1.5-fold with FDR-adjusted p values less than 0.05 were considered significant. Database searches were performed using non-redundant sequences accessible at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) by using the BlastP program.[30] Data have been submitted to the GEO database at NCBI (GSE24413).

Statistical analyses for TNF bioassays and fatty acid profiles. For the TNF bioassays, a minimum of six biological replicates were performed for each condition and analyzed by one-way analysis of variance (ANOVA). Fatty acid profiles were determined with a minimum of three biological replicates (three different cultures from three different initial colonies). All error bars in the figures represent standard deviations.[31]

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Conflict of Interest

J.V. serves as a consultant for Biogaia AB and Group Danone.

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